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Blood miR-21 and miR-26 tailor a good diagnostic model for childhood asthma

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ABSTRACT

This study was to investigate the relationships between blood miR-21/26a with the prevalence and severity of childhood asthma (CAMP). For this purpose, 123 children with allergic asthma (AZ) from June 2018 to June 2020, and 60 contemporaneous healthy children for reference, were enrolled. Lung function was detected using a portable pediatric spirometer and AZ severity was evaluated. Blood samples of admissions were collected to quantify the expression degrees of miR-21 and miR-26a. Logistic regression analysis and model were constructed. Results showed that (1) CAMP had higher MiR-21 expression and lower MiR-26a expression than healthy controls; (2) The severity of AZ, evidenced by FEV1/PV, significantly correlated with miR-21(Y=-3.825X+102.6, P<0.001) and miR-26a (Y=10.43X+54.29, P<0.001); (3) The prevalence of AZ-related to miR-21 (OR=4.180, P<0.001) and miR-26a (OR=0.058, P<0.001) after adjusting for cofounders. (4) the expression levels of miR-21/26a had a good diagnostic potential for AZ (AUC are 0.85 and 0.94, respectively). In conclusion, Blood miRNA-21 and miR-26a are promising biomarkers for the diagnosis and severity of CAMP.

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Introduction

Asthma (AZ), which characterized by reversible airflow obstruction and bronchial hyperresponsiveness, presenting as dyspnea, cough, chest tightness, etc (1,2). Individual variations in immune reactions in AZ can be ascribed to genetic and epigenetic distinctions. MicroRNAs (miRNAs), highly conserved RNA molecules, had no functions of coding amino acids but can control the gene expression process, cleavage, or hinder the expression of mRNAs involved in protein regulation (3). Research indicates that (4), miRNAs may be implicated in the progression of allergic airway disease, and its possible mechanisms may be via regulating inflammation and immunity. miR-21 has been indicated to be involved in inflammation and hematopoiesis and plays a critical role in the development of some allergic conditions (5). Overexpression of miR-21 is associated with the over-activation of allergic cells and may contribute to AZ (6). Deficiency of miR-21 results in a decrease in functional $CD4^+$ T cells, and Th2 cytokines in the airways (7). MiR-26 is the most abundantly expressed miRNA in the lung. MiR-26a inhibits IL-13 expression by directly regulating the 3'UTR of IL-13 transcript, thereby regulating the occurrence and progression of airway allergic inflammation by regulating IL-13 secretion (8). IL-13, a cytokine mainly secreted by Th2 cells, acts as a central regulator of goblet cell hyperplasia, IgE synthesis, mucin hypersecretion, bronchial hyperresponsiveness, chitinase upregulation, and fibrosis under inflammatory conditions in AZ (9,10). Previous animal studies on the role of miRNAs in AZ pathogenesis have led to a deeper understanding.

This study explored the potential of blood miR-21 and miR-26a as promising biomarkers for childhood asthma (CAMP) and its association with AZ severity and possible mechanisms.

Materials and Methods

Study populations

Children with allergic AZ admitted to the People's Hospital of Cangnan and the third hospital of Cangnan between June 2018 and June 2020 were enrolled for assessment of AZ severity and biochemical markers.

Inclusion criteria: (I) Age between 5-16 years; (II) CAMP diagnosis according to the European Respiratory Society clinical practice guidelines; (III) Patients' guardians signed informed consent forms after being fully aware of the research purpose and content.

Exclusion criteria**:** (I) Children with serious heart, liver, or kidney disease; (II) Children with other conditions that are associated with respiratory symptoms, such as chest tightness, and shortness of breath; (III) Recent anti-AZ or allergy medication (3 weeks). Finally, 123 eligible patients were enrolled (the People's Hospital of Cangnan; the third hospital of Cangnan (83)).

Additionally, 60 contemporaneous healthy children from the third hospital of Cangnan were enrolled as a reference.

Lung function assessment

Forced expiratory volume (1s) (FEV1) was assessed in children using a portable pediatric spirometer after discontinuing short-acting bronchodilators at least eight

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hours before testing. In lung function tests, the predicted value (PV) is expressed as a percentage. AZ Severity was defined as mild (FEV1/PV, > 80%), moderate (FEV1/PV, 60%-80%), or severe (FEV1/PV, <60%).

Serum samples collection

Collect peripheral blood samples (4ml) from children under sterile conditions and divide them into two parts: collect 2 ml of sample into tubes coating with EDTA and centrifuge the tubes for 10 min; While transferring the plasma phase carefully to an RNase-free tube and centrifuge again for 10 min, serum was prepared by centrifuging the remaining 2 ml of blood at room temperature for 10 min 1000 ×g.

Biochemical index measurement

RNA was extracted using the miRNeasy kit (Qiagen, Hilden, Germany) from the plasma samples. cDNA was reversely transcripted using the miScript II RT Kit (Qiagen, Germany), the SYBR Green detection kit, and the miScript Primer detection kit (Qiagen, USA), on the Stratagene Mx3000p system. T Zhejiang Gloucester Biotechnology Company designed and synthesized the specific primers for miRNA-21 and miR-26a RT-qPCR The primers for MiR-26a are: forward 5'-GCAGTGAGGTAGTAGGTTG-3' and reverse 5'- GGTCCAGTTTTTTTTTTTTTTTTAA-CTATAC-3', for miR-21 are: forward 5'-GATACTCA-TAAGGCACGCGG-3' and reverse 5'-GTGCAGGGTC-CGAGGT-3'. And the primers. The housekeeping gene miRNA SNORD68 was used for internal control, and its primer sequences were: forward 5-CTCGCTTCGG-CAGCACA-3 and reverse 5-AACGCTTCA CGAATT-TGCGT-3. Expression of miR-21 and miR-26a relative to housekeeping genes was calculated by the $2^{\Delta\Delta C}$ method.

Follow the manufacturer's instructions, Immunoassays for IL-13 levels in serum using a commercial ELISA (Bender MedSystems, Germany).

Statistical Analysis

Quantitative and qualitative data were expressed as (median ± IQR) and number (percentage), respectively. An analysis of differences between two or more groups was conducted using the Mann-Whitney U test or the Chi-square test. Logistic regression and Spearman correlation analysis were used to explore the relationships between miRNAs and the prevalence and severity of AZ. All analyses were conducted in SPSS 20.0 and P<0.05 was identified as statistically significant.

Results

Baseline characteristics.

The median age of the AZ group and healthy controls is 10.1 and 10.4 years old respectively. As shown in Table 1, there was no difference in age or sex between CAMP and control groups, however, family AZ history, and FEV1/PV were different between the two study groups (P<0.001), Compared with healthy children, the miRNA-21 expression level and the blood level of IL-13 in CAMP were increased (P<0.001), while the expression level of miR-26a in CAMP was reduced than that in healthy children (P<0.001) (Table 1).

Predictive effects of serum micro-RNAs on ZA.

As shown in Table 2, univariable logistic regression analysis found that both and miR-26a have a significant association with the prevalence of AZ (miR-21: OR=3.717, P<0.001; miR-26a: OR=0.087, P<0.001). After adjusting for confounders, including age, sex, and family AZ, the significance remained (miR-21: OR=4.180, P<0.001; miR-26a: OR=0.058, P<0.001)

Univariate ROC analysis found that the AUC of miR-NA-21 in distinguishing mild to moderate AZ markers was 0.85 (95%CI: 0.79-0.90, P=0.027), at cutoff value > 3.5, the sensitivity and specificity of miRNA-21 to distinguish CAMP from control children were 83% and 89%, respectively. Additionally, the AUC of miR-26a in distinguishing asthmatic patients from healthy subjects was 0.93 (95%CI: 0.89-0.97, P=0.018), with a sensitivity of 84% and a specificity of 92%, and the cut-off value of MiR-26a < 2.01. After adding other variables in model 2, the AUC of the final predictive model to ZA reached 0.89 and 0.95 for miR-21 and miR-26a respectively (Figure 1).

Blood micro-RNAs according to AZ severity

As shown in Figure 2, as the severity of AZ progressed,

Table 1. Baseline characteristics.

	HC (n=60)	MiA (n=59)	MoA (n=33)	SA (n=31)	P value#
Demographic characteristics					
Age (years)	10.4 (7.9-12.2)	10.8(8.5-11.8)	9.2 (7.2-11.3)	9.9 (9.0-11.8)	0.781
Sex (n, %)					0.621
Female	25(41.7%)	30(50.8%)	20(60.6%)	14(45.2%)	
Male	35(58.3%)	29(49.2)	13(39.4%)	17(54.8%)	
Family Asthma history (n, %)	19(31.7%)	47(79.7%)	27(81.8%)	26(83.9%)	<0.001
Biochemical indices					
Il-13 (pg/ml)	5.5 (4.0-6.7)	10.9 (8.3-13.7)	16.4(12.4-18.0)	18.6 (15.9-20.7)	<0.001
miR-21 (dTC)	3.6 (3.2-3.9)	4.6 (3.7-5.9)	7.3(4.9-8.1)	9.2 (7.9-12.6)	<0.001
miR-26a (dTC)	3.9 (3.3-4.4)	2.6 (2.1-3.1)	1.6 (1.3-2.1)	1.1 (1.0-1.3)	<0.001
Lung function parameter					
FEV1/PV (%)	98.9 (98.3-99.4)	82.1 (82.1-83.5)	71.5 (68.4-75.3)	55.4 (53.0-57.5)	<0.001

Values were presented as Median (IQR). # P of M-U test or Chi-square test between HC and asthma group. Abbreviations: HC, healthy controls; MiA, mild asthma; MoA, moderate asthma; SA, severe asthma; FEV1/PV, forced expiratory volume in the first second to the predictive value ratio.

Table 2. Regression analysis according to the prevalence of AZ.

		miR-21	P value	miR-26a	P value
Model 1	Crude OR (95%CI)	3.717 (2.236-6.178)	<0.001	0.087 (0.043-0.178)	<0.001
	AUC (95%CI)	0.854 (0.794-0.901)	0.027	0.936 (0.890-0.967)	0.018
Model 2	Adjusted OR# (95%CI)	4.180 (2.374-7.359)	<0.001	0.058 (0.024-0.137)	<0.001
	AUC (95%CI)	0.887 (0.832-0.929)	0.023	0.949 (0.906-0.976)	0.017

#Adjusted by age, sex, and Family AZ history. Model 1: single micro-RNA; Model 2, model 1 + age +sex+ family AZ history.

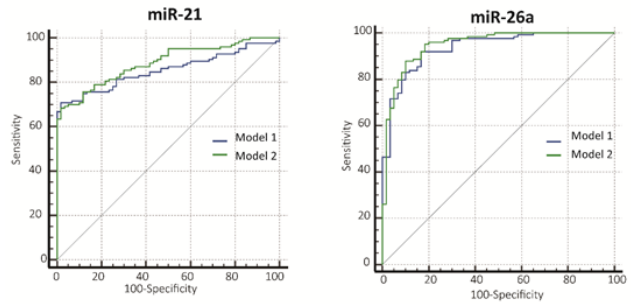

Figure 1. The ROC curves of the two models. Model 1: single micro-RNA; Model 2, model 1 + age +sex+ family AZ history.

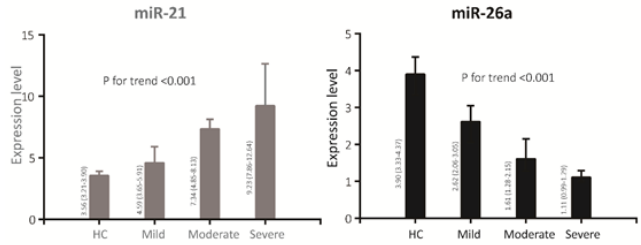

Figure 2. Blood micro-RNAs according to AZ severity. Values were presented as Median (IQR).

there is an increased expression trend of miR-21 (P for trend <0.001) and decreasing trend of miR-26a (P for trend <0.001). The miRNA-21 expression was negatively correlated with the level of FEV1/PV (Y=-3.825X+102.6, R square=0.508, P<0.001), while it was positively correlated with FEV1/PV (Y=10.43X+54.29, R square=0.655, P<0.001) (Figure 3).

Blood micro-RNAs and inflammation

Spearman analysis found that the expression level of miR-21/26a were both significantly related to IL-13 (miR-21: r = 0.349, P<0.001; miR-26a: r = -0.270, P<0.001) (Figure 4). Linear regression analysis further uncovered that the miR-21 expression was positively correlated with the IL-13 level (Y=1.083X+5.306, R square=0.284, P<0.001), and the miR-26a expression negatively correlated with the IL-13 level (Y=-3.129X+19.45, R square=0.411, P<0.001).

Discussion

Micro-RNAs are small and medium-sized regulatory RNAs with important effects on various cellular processes and cell fate determination (11-13). In AZ, it has been suggested that miRNAs can modulate allergic immune responses via Th2 differentiation and differentiation (14-16).

The research reported, the expression of miR-21 was upregulated in the lungs of mice stimulated with ovalbumin (OVA) compared to unstimulated mice, pulmonary eosinophils and mucus production were reduced in OVA-treated miR-21 knockout (KO) mice (17-19), furthermore, OVA-treated miR-21 KO mice showed markedly reduced bronchial hyperresponsiveness. MiR-21 may play a key role in Th2-related allergic conditions by regulating Th2 cells and cytokines (20-22). The miR-21 expression in CAMP was raised than that in healthy control children, at the cutoff value of 3.5, the sensitivity and specificity of miRNA-21 to distinguish CAMP from control children were 83%

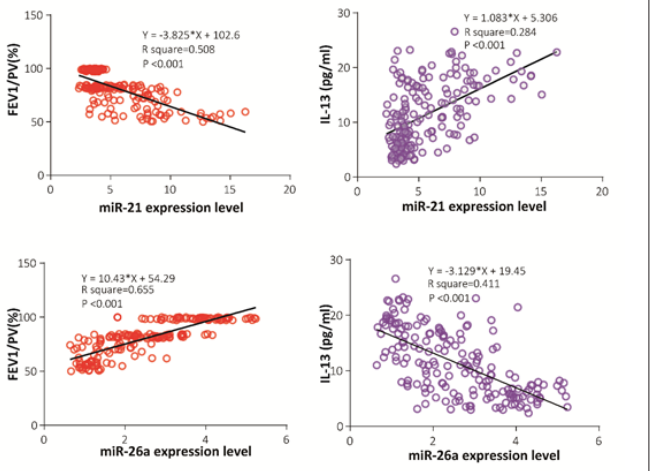

Figure 3. Linear correlation analysis of miR-21 and miR-26a with serum IL-13 levels and FEV1/PV.

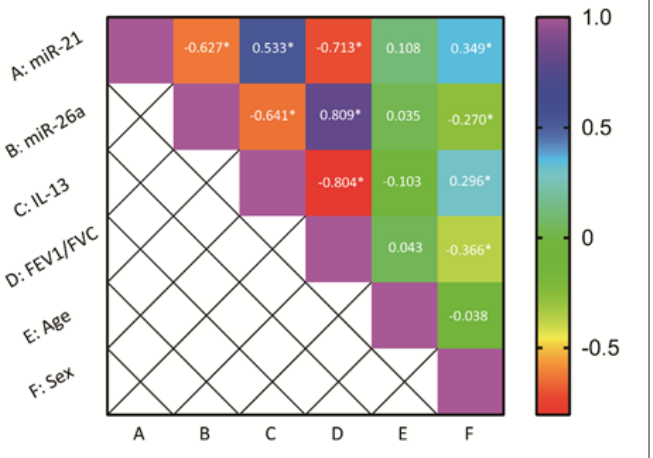

Figure 4. Spearman analysis. The intensity of the color represents the r-value of Spearman's analysis between the relative abundance of thyroid indexes and other factors. The r value was displayed in each color block. *P<0.05.

and 89%, respectively. The expression level of miR-21 in CAMP was higher. Further, miRNA-21 could predict AZ severity (AUC = 0.85, P = 0.027). The results suggest that miR-21 plays an important role in AZ pathogenesis and immune imbalance, and can be used for AZ severity diagnosis and prediction. The literature points out (23-25), it has an important regulatory role in allergic inflammation mediated by ILC2 and IL-13 and thus is involved in allergic airway inflammation, in addition to AZ, the miR-21 expression was increased in patients with allergic rhinitis with positive nasal mucosa and skin prick tests. Previous researches (26) research showed that airway epithelial cells and serum miR-26a expression is reduced in AZ patients. In addition, recent studies have pointed out (19), that the miR-26a expression was reduced in exhaled air condensate of AZ patients compared with controls, eight miRNAs were detected in asthmatic individuals' bronchoalveolar lavage fluid by miRNA array analysis, of which miR-26a was downregulated. The expression level of miR-26a in CAMP was reduced than that in control children. With a sensitivity of 84% and a specificity of 92%, miR-26a was able to effectively distinguish AZ from healthy controls with a cut-off value < 2.01 (AUC = 0.94, P = 0.018). Therefore, MiR-26a plasma levels can be used as a diagnostic biomarker in AZ patients. Furthermore, severe AZ patients showed reduced miR-26a expression compared to mild and moderate cases. Therefore, AZ severity can be differentiated by MiR-26a expression. Previous studies (27,28) used small interfering RNAs targeting miR-21 to effectively reduce inflammation, eosinophils, and Th2 cytokines levels, cytokine levels. Serum IL-13 was analyzed by ELISA, and the serum IL-13 level in AZ patients was raised than that in control children, and it was correlated with the severity of the disease. Spearman analysis showed that the miRNA-21 was positively correlated with IL-13 and negatively correlated with FEV1/PV, while the miR-26a had the opposite trend. Studies indicate that miR-7 targets IL13 3'UTR inducing its inhibition and AHR.

Conclusively, the expression of miR-21 was increased in the peripheral blood of CAMP, while miR-26a was decreased, and its levels were correlated with disease severity, IL-13 levels, and lung function parameters, indicating that blood miR-21 and miR-26a were promising biomarkers for CAMP severity and diagnosis.

Acknowledgments

Ethics approval and consent to participate

The Ethical Decision Committees of the People's Hospital of Cangnan and the third hospital of Cangnan have approved the study. And all patients agreed to participate in the study and use their clinic data and information for research purposes.

Consent for publication

All participants agreed to publications related to this study.

Availability of data and material

Data and material can be shared with the consent of the corresponding authors.

Competing interests

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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Authors' contributions: LiDang Lu and GuoZhong Zheng were responsible for data collection, statistical analysis and writing the paper. YuanJing Lin provided resources and designed the study.

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